PREPARATION AND ANTIGENICITY OF M PROTEIN RELEASED FROM GROUP A, TYPE 1 STREPTOCOCCAL CELL WALLS BY PHAGE-ASSOCIATED LYSIN

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Among Group A streptococci, the M antigen has been designated the basis for type specificity (1), and is the most important known determinant of the virulence of these organisms (2). Furthermore immunity to infection with Group A streptococci is largely type-specific (3, 4), and principally dependent upon the presence of antibodies directed against the M antigen. This antigen was extracted from a Type 1 organism and partially purified by Lancefield and Perlmann (5) and many of its physical and chemical properties elucidated. The method of extraction, in a boiling water bath at pH 2, resulted in a hapten-like protein, which was serologically reactive but antigenically poor.

The present paper presents a method of preparation and partial purification of Type 1 M protein at physiological temperature and hydrogen ion concentration, utilizing the phage-associated lysin recently described by Maxted (6) and Krause (7). Antigenicity of this preparation has been determined in rabbits by means of precipitin, bactericidal, mouse protection, and long chain tests.

Materials and Methods

Media.—(*a*) Horse-meat infusion broth with 2 per cent neopeptone was obtained as a standard preparation from the National Institutes of Health Media Section.

(*b*) Todd-Hewitt broth was prepared as originally described (9) except that horse-meat was used instead of beef hearts.

(*c*) "Dialysate media" was prepared by the method of Krause (7).

(*d*) Non-protein semisynthethic media consisted of two parts: Eagle's basal medium (10) and peptone dialysate. The latter was prepared with slight modification of the method of Dole (11). To make 2 liters of peptone dialysate: 250 gm. of Pfanstiehl peptone + 10 gm. of powdered charcoal are heated in 400 ml. of distilled water to 80°C. for 15 minutes and filtered while hot through hyflow super-cel (Johns-Manville Sales Corp., New York, diatomaceous earth). The filtrate is cooled to room temperature and dialyzed twice against 500 ml. of distilled water in a shaking dialyzer for 18 hours. The dialysates are combined and reabsorbed with 50 gm. of charcoal at 80°C. for 15 minutes and filtered again through super-cel. After cooling, 9 gm. of NaCl are dissolved in the filtrate and the pH adjusted to 7.5.

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The volume is brought to 2 liters, and the dialyzed peptone is then filtered through a Selas 0.03 filter and stored at 4°C. Four liters of the complete non-protein semisynthetic media were made by adding 1 liter of peptone dialysate to 3 liters of Eagle's basal media and finally adding 20 ml. of a sterile 50 per cent glucose solution.

Streptococci.—Three strains of Type 1 streptococci were used. Strains T1/SS70L1[1] and T1/155/4[2] were stored in the lyophilized state, cultured as needed in horse-meat infusion broth with neopeptone, and serially passed through mice to enhance or maintain virulence. After 124 passages, the LD_{50} of T1/SS70L1 for 20 gm. white mice reached 10 to 50 organisms: daily subcultures from sheep blood agar plates were then employed and new cultures were taken from lyophilized portions whenever loss of virulence was shown by inability to grow in normal human blood (8). Cell walls for preparation of lysin M protein were obtained from this strain. The LD_{50} of T1/155/4 was between 5 and 10 organisms as received, and only occasional mouse passage was required to maintain this level: this strain was employed in bactericidal, mouse protection, and long chain tests for type-specific antibody. Strain T1/2788 was obtained as phenolized bacterial cells[3] and was the source of acid M protein.

Heterologous type strains, similarly maintained were used in testing specificity of antisera: they were SF 42 (Type 12),[4] S23/94/3 (Type 14),[4] and T23 (NCTC 8301) (Type 23).[5]

The propagating strain for C_1 bacteriophage was a strain of Group C streptococci designated 26RP66.[6]

Bacterial cells for absorption of standard type-specific antiserum were obtained from Todd-Hewitt broth cultures of streptococcal strain C203:[4] this contains Type 3 M antigen and Type 1 T antigen.

Preparation of Cell Walls.—Streptococci were grown in 4 liter lots of modified Todd-Hewitt broth at 37°C. and collected in a Servall continuous flow centrifuge after overnight incubation. The streptococcal cells, suspended in distilled water, were disrupted in the Mickle disintegrator after the method of Salton (20). A drop of tri-*n*-butyl phosphate was necessary in each shaking chamber to prevent foaming. Cell disruption was monitored by the use of phase microscopy. Following disruption, the cell walls were washed three times in 0.1 M potassium phosphate buffer at pH 7.5, followed by exposure to crystalline ribonuclease[7] in final concentration of 0.01 mg./ml. for 5 hours at 37°C. The cell walls were again washed three times in distilled water and resuspended in buffered saline.

Preparation of Acid Extracted M Protein.—Partially purified M protein was prepared from T1/2788 streptococcal cells by the method of Lancefield and Perlmann (5). Boundary electrophoresis of this preparation at p H 8.6 revealed a single peak with sufficient boundary spreading to suggest some heterogeneity. M protein reacted with absorbed antiserum at concentrations as low as 5 γ/ml. and was in all respects similar to the preparation described by the earlier workers. No attempts at further purification were made.

Preparation of Type 1 "T" Protein.—A crude preparation of Type 1 T protein was extracted by pepsin from a culture of C203 organisms by the method of Lancefield and Dole (16). Approximately 1.5 liters of an 18 hour broth culture were centrifuged and the cells

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[1] Obtained from Dr. Elaine Updyke, Laboratory Branch, Communicable Disease Center, Chamblee, Georgia.

[2] Obtained through the courtesy of Dr. Rebecca C. Lancefield of The Rockefeller Institute.

[3] Kindly supplied by Dr. C. P. Hegarty of Merck, Sharpe and Dohme, West Point, Pa.

[4] Obtained through the courtesy of Dr. Rebecca C. Lancefield of The Rockefeller Institute.

[5] Obtained originally from Dr. D. G. Fleck, Streptococcal Reference Laboratory, Colindale, London, and kindly supplied to us by Miss Alba E. Colon, formerly of the Department of Microbiology, University of Miami School of Medicine.

[6] Kindly supplied by Dr. Richard M. Krause of The Rockefeller Institute.

[7] General Biochemicals Inc., Chagrin Falls, Ohio.

suspended in 15 ml. of 0.85 per cent saline to which N HCl was added to adjust the reaction to pH 2.5. To one-half of the suspension 5 ml. of 2 per cent pepsin (Difco 1:10,000) was added. To the other half, 5 ml. of the same pepsin solution, previously inactivated by heat, was added. After overnight incubation at 37°C. both suspensions were centrifuged and the supernatants tested with Type 1 T antiserum. The tube containing active pepsin proved reactive; the control was not. No further purification of T protein was undertaken.

Preparation of Group A Polysaccharide.—Purified polysaccharide[8] was prepared by the method of McCarty (17).

Bacteriophage.—Strain C_1 bacteriophage[8] was stored in horse-meat infusion broth at 4°C. The Gratia soft agar method (12), using agar plates prepared according to Krause (7), was employed for counting phage particles initially. In later experiments a more convenient method was used. It consisted of pipetting 0.2 ml. of a log phase culture of the propagating strain onto the center of a sheep blood agar plate. The culture was evenly distributed over the surface of the plate with a bent glass rod and then allowed to dry. A measured drop (0.02 ml.) of a phage dilution was pipetted onto each quadrant, and the plate incubated at 37°C. for 12 of to 18 hours. Phage plaques appeared as holes in the bacterial lawn. The titer of phage particles when assayed by this method was within 0.3 logs of the titer using the soft agar method.

Production of Phage Associated Lysin.—Lysin is a protein prepared from phage lysates by precipitation with ammonium sulfate (21). To avoid precipitating other proteins present in most media, a non-protein medium seemed appropriate. Protein-free dialysate broth, media (*c*) above, was slightly modified and used for this purpose by Krause. The beef heart–extract portion of this media was time-consuming to produce: consequently the growth characteristics of the C_1 propagating strain 26RP66 in different media were determined (Fig. 1). Eagle's basal media with glutamine as a nitrogen source did not support growth; when peptone dialysate was added to the basal medium, however, growth was comparable to the standard protein broths. The materials for this medium are considerably more expensive than those for dialysate broth, but availability and a more uniform product have dictated its use.

The method used to isolate lysin from fresh phage lysates is essentially that of Krause (7) with some modification. Semisynthetic non-protein media is inoculated with approximately $\frac{1}{80}$ its volume of an overnight culture of the propagating strain (26RP66) of Group C streptococci. The culture is incubated at 37°C. until it reaches an optical density of 0.15 to 0.2 in the Coleman Jr. spectrophotometer measured at 530 mμ. This density represents approximately 5×10^8 organisms/ml. The culture is then infected with C_1 bacteriophage and incubated at 37°C. until complete lysis occurs. Multiplicity of phage particles should be close to 1 to insure complete lysis within 1 hour. Part of the resulting lysate is removed and heated to 45°C. for 15 minutes to destroy the phage-associated lysin produced. The heated portion is then phage-titered and used for inoculum in the next batch. The remainder of the unheated lysate is stored at 4°C. for 2 to 3 days. Lysates from several days are then combined and precipitated at 0.5 saturation with ammonium sulfate. The precipitate is allowed to stand at 4°C. for 2 to 3 days and then collected by high speed (20,000 to 30,000 *G*) centrifugation. The supernate is discarded and the button is then taken up in buffered saline at pH 7–7.5. After clarification of the resulting solution by centrifugation at 4,000 *G* for 30 minutes, the supernate is removed and saved. The button is washed twice with buffered saline and the washings after centrifugation are combined with the initial supernate. The phage particles are partially removed by two successive centrifugations in the Spinco ultracentrifuge at 78,000 *G* for 2 hours. The resulting lysin solution is clear, faintly yellow in color, and contains 10^4 to $10^{5.5}$ particles per ml. The sedimented phage particles are resuspended in horse-meat infusion

[8] Kindly supplied by Dr. Richard M. Krause.

broth, clarified by centrifugation at 4,000 *G* for 15 minutes, and filtered through a Selas 0.03 filter to be used as phage inocula in later batches. The lysin solution is used immediately or frozen and stored at −70°C.

Electrophoresis.—The electrophoretic studies were carried out at 2°C. in an aminco-Stern apparatus. Protein solutions (1 per cent) were dialyzed against large volumes of sodium veronal buffer for 2 days prior to electrophoresis; the same buffer was used in the analysis.

Antisera.—To produce a standard Type 1 antiserum, rabbits were immunized by repeated intravenous injections of whole heat-killed streptococci (T1/SS70L1) for a 5 week course

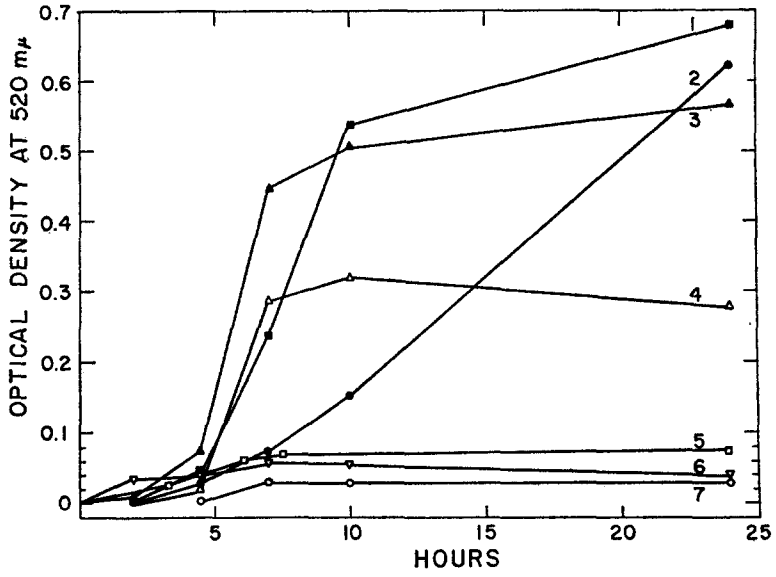

FIG. 1. Growth of strain 26RP66 (Group C) streptococci in various media:

1. Modified Todd-Hewitt broth.
2. Beef heart extract + dialyzed peptone ("dialysate media" of Krause).
3. Eagle's basal medium + peptone dialysate.
4. Horse-meat infusion broth + 2 per cent neopeptone.
5. Dialyzed peptone.
6. Eagle's basal medium.
7. Eagle's basal medium + glutamine (final concentration, 0.03 per cent).

(13). Two weeks after the last injection they were tested for the presence of type-specific antibody by direct and indirect bactericidal tests (14). When the presence of antibody was confirmed, a large bleeding from the heart was performed and the serum was separated under sterile conditions, dispensed in 1 ml. amounts, and frozen at −20°C. Only one vial was thawed for use at any one time and was then stored at 4°C. Repeated freezing and thawing tended to reduce bactericidal activity. No preservatives were used and serum was not heated.

Following absorption of standard type-specific antiserum with streptococcal strain C203, it proved non-reactive with Type 1 T protein.

Type 1 anti-T antiserum was prepared by the method of Lancefield (15). Rabbits were immunized with heat-killed streptococci of strain C203, in which the M antigen was destroyed

by 2 hour digestion with pepsin. Antibodies other than the homologous anti-T precipitins were removed by selective absorption of the immune sera with heterologous strains known to contain no serologically related T antigens.

For routine grouping and typing, absorbed rabbit antisera supplied by CDC[9] were used.

Immunological Techniques.—Precipitin tests were performed with the capillary tube technique (18). The detailed method used for bactericidal tests (both direct and indirect) has been described previously (8, 19) and summarized below. In the direct test, an inoculum of organisms in 0.1 ml. of broth is introduced into a 7 × 10 mm. tube with 0.3 ml. of the freshly drawn heparinized[10] blood of the animal to be tested. After rotation of the stoppered tube for 3 hours at 37°C., 0.1 ml. is removed and plated to determine growth or the lack of growth of the inoculum. In the indirect test a *normal* human serves as the donor; *i.e.*, donor blood alone will not inhibit the growth of the inoculated microorganisms. To 0.05 ml. of serum from the animal to be tested, 0.3 ml. of normal, freshly drawn donor blood is added, and 0.1 ml. of organisms introduced. After rotation for 3 hours, a 0.1 ml. sample is plated to determine number of surviving bacteria; in the presence of a suitable donor blood and type-specific antibody, there is a reduction in the number of bacteria inoculated. Conversely, in the absence of type-specific antibody, the inoculum increases by a factor of 32 to 64. Positive and negative control sera were used in each test. For convenience in comparing the results of several bactericidal tests the bactericidal index was used. This is a ratio of the inhibition of growth in the presence of a test blood to the inhibition of growth in normal blood. The detailed derivation of the formula has been reported elsewhere (14). If the (index) ratio is 1, then growth in the test and control tubes is the same. An index of 100 means that growth in the control tube was 100 times greater than that in the test. Indices were considered as follows: 0 to 25, indeterminate; 25 to 50, weakly positive; 50 to 200, positive; 200 to 500 or greater, strongly positive.

Stollerman and Ekstedt have reported an effect on chain length of streptococci grown in immune serum (19). The lengthening of the streptococcal chain is a type-specific phenomenon, sensitive to relatively small amounts of antibody. The detailed procedures used in long-chain and mouse-protection tests are described in the individual experiments.

Experimental Animals.—White, male rabbits, weighing 2 kg. each, were obtained from the Animal Production Unit of the National Institutes of Health. They were supplied from a closed colony started 23 years ago with 12 original rabbits of the New Zealand White strain

EXPERIMENTAL

Preparation and Reactions of Lysin-Digests.—The suitability of a particular strain of streptococci for lysis by phage-associated lysin varies considerably, but certain conditions will enhance the lysis of all strains (7). Most important is the maintenance of lysin in the reduced form.

Lysin prepared from 30 liters of phage lysate in a final volume of 150 ml. of buffered saline was added to a flask containing cell walls prepared from 50 liters of culture in a volume of 200 ml. of buffered saline at pH 7.4. To the reaction mixture 0.5 ml. of thioglycolic acid,[11] neutralized with N NaOH to pH 7.4, was added. The air in the flask was then displaced with nitrogen and the flask stoppered and incubated at 37°C. for 6 hours with mild agitation. During the course of the incubation, the density of the suspension lessened noticeably but did not

[9] Diagnostic Reagents Section, Communicable Disease Center, United States Public Health Service, Chamblee, Georgia.

[10] Heparin sodium, Abbott Laboratories, North Chicago, 1000 units/cc., was used in the proportion of 0.1 ml. for 10 ml. of rabbit blood, or 0.1 ml. for 20 ml. of human blood.

[11] Matheson Company, Inc., East Rutherford, New Jersey.

approach clarity. After incubation, the contents of the flask were centrifuged at 20,000 *G* for 30 minutes and the clear lysin digest decanted from the debris. The digest was tested for the presence of C polysaccharide and M protein with appropriate antiserum by the capillary precipitin technique. The precipitin reaction with Group A antiserum was immediate and strong. With Type 1 absorbed antiserum a fine haze appeared after incubation at 37°C. for 2 hours; it did not coalesce following overnight incubation at 4°C. But, if the digest was first dialyzed against 0.01 M phosphate at pH 7.0, the precipitin reaction with Type 1 antiserum was prompt and strong.

To determine the effects of various salt concentrations on the reactivity of the lysin digest with type-specific antiserum, samples of digest in 0.01 M buffer were diluted with equal parts of several different buffers and then set up in capillary precipitin tubes against type-specific antiserum. For comparison, acid-extracted, partially purified M protein was tested similarly. Table I shows the results.

TABLE I

Precipitin Reactions of Lysin Digest and Acid-Extracted Type 1 M Protein at Various Salt Concentrations with Type-Specific Antiserum

Antiserum	Antigen in:	0.01 M buffer	0.2 M buffer	0.4 M buffer	0.6 M buffer	1 per cent saline
Absorbed Type 1	Lysin digest	++++	+++	++	±	±
	Acid M protein*	++++	++++	++++	++++	++++

* Concentration of acid-extracted M protein varied from 50 γ/ml. to 5 mg./ml. with similar results.

Reactivity of lysin digest with type-specific antiserum varied inversely with the salt concentration of the buffer; the precipitin reactions of acid-extracted M protein, tested in concentrations varying from 50 γ/ml. to 5 mg./ml., were not so affected, suggesting that the difference in reactivity of acid M protein and lysin digest is a qualitative and not a quantitative one.

Purification of Lysin Digest.—

The crude digest was partially purified on a diethylaminoethyl-cellulose column. Column material was first cycled four times with N HCl alternating with N NaOH. It was then washed with 0.01 M phosphate buffer at pH 7 in a 4 liter beaker and allowed to sediment for 1 hour. The supernate which contained small fibers was decanted and the washing repeated three times. The suspension of the ion exchange material was then poured into a 20 mm. column and allowed to pack by gravity. Approximately 1 liter of 0.01 molar buffer was run through the column by gravity alone. The effluent was then checked to confirm the pH of 7 and absence of 280 mμ absorbing material.

The crude lysin digest, after dialysis against 0.01 molar phosphate buffer at pH 7, was run into the column at 6 drops a minute. After loading the column, 0.01 molar phosphate buffer was again run through at the same rate. The elution chromatogram obtained is illustrated in Fig. 2. The first peak represents that material which did not adsorb on the column. It has a broad flat curve in the

ultraviolet absorption spectrum at 260 mμ as illustrated in Fig. 3 (*A*). When the column effluent contained no more protein, the buffer was changed to 0.1 molar phosphate in physiological saline at pH 7.0. Equilibration between the protein on the column and in the buffer takes place slowly. For this reason, the flow rate of the second buffer was reduced to 1 drop/2 minutes. The ultraviolet absorption spectrum of the second peak as shown in Fig. 3 (*B*) is a protein absorption curve with a minimum at 255 mμ and maximum at 280 mμ.

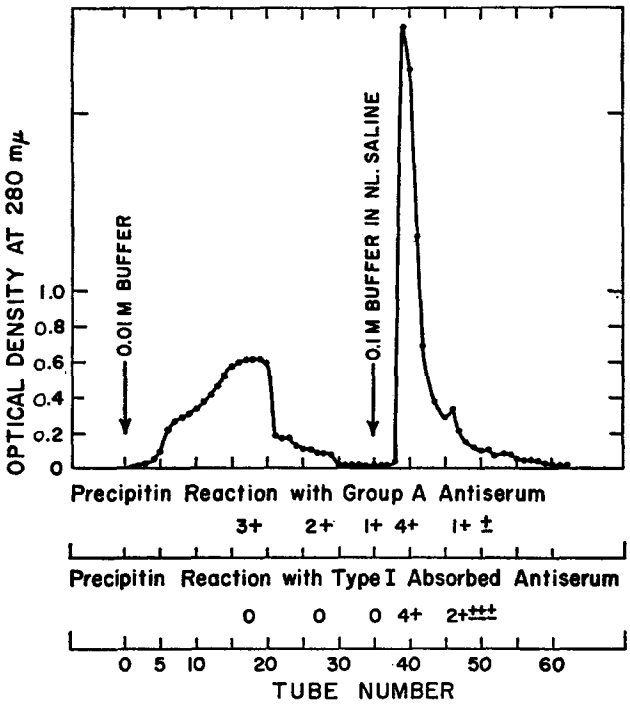

FIG. 2. Chromatogram and precipitin reactions of cell wall digest eluted from a DEAE cellulose column.

The contents of the tubes to be tested by the precipitin technique were first dialyzed against 0.01 molar phosphate buffer to negate the salt effect on the precipitin reaction. The group-specific carbohydrate was not adsorbed on the column and appeared along with the first peak; the concentration of this carbohydrate apparently diminishes but does not disappear. In several experiments, large volumes of 0.01 molar phosphate buffer were used to try and wash out all the carbohydrate; this was not possible without eluting the protein at the same time.

With the change of buffer, the second peak was eluted and is seen (Fig. 2)

to contain the type-specific reacting material. Of interest is the increased reactivity of the material eluted in the second peak with Group A antiserum.

In one experiment the material in both peaks was concentrated and tested for the presence of phage-associated lysin. No activity could be found in either sample when lysis of a standard suspension of streptococcal cells in the presence of thioglycolate was attempted.

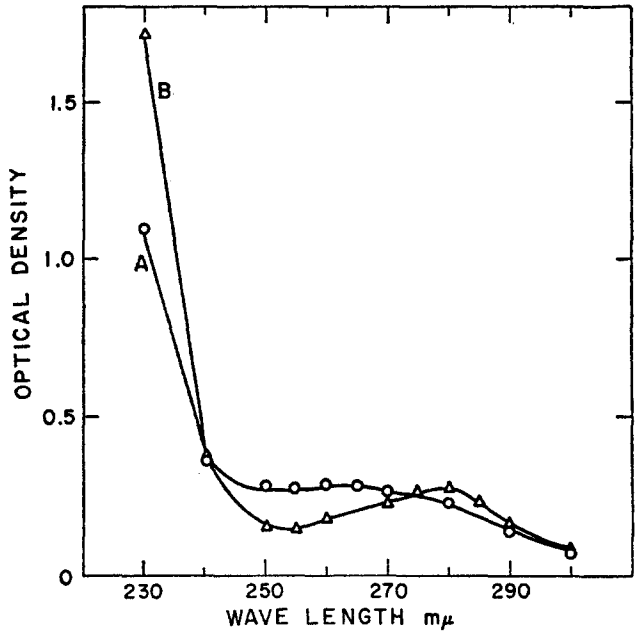

FIG. 3. Ultraviolet absorption spectra of material eluted in first peak (*A*) and second peak (*B*) from DEAE cellulose column.

Tube Nos. 34 to 60 (Fig. 2) were combined and concentrated by forced evaporation in a dialysis bag. This material is called "crude-lysin M protein." It was further purified by repeated precipitation with ammonium sulfate at 0.6 saturation. To enhance precipitation the reaction of the solution was adjusted to pH 6 with 6 N HCl. The final precipitate was taken up in phosphate buffer and dialyzed overnight against 0.02 molar phosphate buffer. For convenience it is designated "lysin M protein" to distinguish it from material prepared by the method of Lancefield and Perlmann which is called "acid M protein."

Composition of Lysin M Protein.—To determine sensitivity of the lysin M preparation to proteolytic enzymes and to acid extraction at 95°C., the following experiments were performed:

The lysin M protein preparation was diluted 1:2 in phosphate buffer and 0.5 cc. was added to an equal volume of 0.1 per cent commercial trypsin.[12] The mixture was incubated for 2 hours at 37°C. and 0.5 cc. of 0.2 per cent solution of soy bean inhibitor[12] added.

A second sample of the preparation was acidified with N/5 HCl and placed in a boiling water bath for 15 minutes, cooled, and neutralized with N/5 NaOH. The reaction of a third sample was adjusted to pH 2.5 with N/5 HCl, and pepsin[13] was added to final concentration of 1 mg./ml. This sample was incubated for 2 hours at 37°C., cooled, and neutralized with

TABLE II

Precipitin Reactions of Lysin M Protein Altered by Treatment with Acid and Proteolytic Enzymes

Sample		Type 1 M antiserum	Type 1 T antiserum	Group A antiserum
No.	Treatment			
I	Trypsin	−	++	+++
II	pH 2, 95°C.*	+++	−	+++
III	Pepsin	−	++	+++

* Specificity of the non-enzyme-treated Type 1 lysin M protein was shown by its failure to react in the precipitin test with antisera for Groups B, C, D, E, F, G, H, K, L, M, and N: or with antisera for Types 3, 4, 5, 6, 8, 11, 12, 13, 14, 15, 17, 18, 19, 23, 24, 25, 26, "28", 30, 31, 32, 33, 36, 37, 39, 40, 42, 43, and 46.

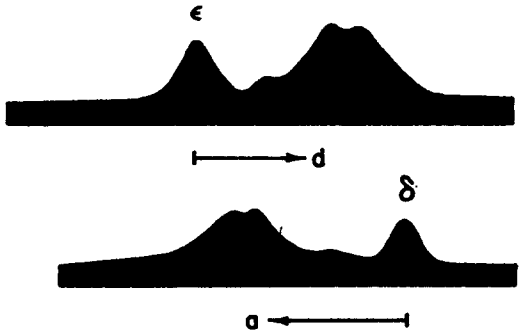

FIG. 4. Electrophoretic pattern of a 1 per cent solution of lysin M protein. Electrophoresis was carried out in a sodium veronal buffer at pH 8.6, at ionic strength = 0.1, for 49 minutes at a potential of 1200 volts, and current of 10 ma.

N/5 NaOH. The volumes of all samples were adjusted with 0.01 M phosphate buffer, so that the final concentration of lysin M was the same in all. The three samples were then tested with type-specific absorbed antiserum, Type 1 T antiserum, and Group A antiserum. The results of this experiment are reported in Table II.

Proteolytic enzymes completely destroyed reactivity of the samples with type-specific antiserum. Exposure to 95°C. for 10 minutes at pH 2 did not

[12] Nutritional Biochemicals Inc., Cleveland; 1:300.

[13] Pepsin, Difco Laboratories, Detroit; 1:10,000.

affect the typing reaction; however, reactivity with T antiserum was destroyed by this treatment. Similarly treated material in 0.01 M phosphate buffer did not react with absorbed heterologous group and type antisera (*cf.* footnote, Table II). All samples reacted with Group A antiserum.

The lysin M preparation thus appears to contain not only M and T proteins but group-specific C polysaccharide as well.

Electrophoresis.—The electrophoretic analysis of lysin M protein was carried out in a veronal buffer, ionic strength = 0.1, at pH 8.6 with a potential of 1200 volts and current of 10 ma. The patterns obtained, Fig. 4, show a partial separation of 3 components, all of which migrate toward the anode. No further isolation or identification of the components was attempted.

Antigenicity.—

A total of ten rabbits (numbered 1-1 to 2-0) was immunized with preparations containing lysin M protein. Concentration of antigen protein was calculated from the optical density at 280 mμ in the Beckman spectrophotometer by reference to a protein standard. Rabbit 1-1 received a total of 1 mg. of crude[14] lysin M protein in complete Freund's adjuvant (22) by intradermal injection at two sites on the abdomen, followed in 2 weeks by 0.5 mg. intradermally in complete adjuvant. Rabbits 1-2 to 1-4 received a similar course of the same antigen in saline. Rabbits 1-5 to 2-0 received a total of 1.0 mg. of lysin[15] M protein in saline at 2 intradermal sites, followed in 2 weeks by 0.5 mg. of the same material intradermally. Rabbit 1-9 died, 1 week after the onset of immunization, of an unrelated respiratory infection. No local reaction was apparent at the site 36 hours after injection of lysin M protein in saline

Antibody to M protein was determined by: (*a*) precipitin tests, using a 0.1 per cent solution of acid M protein as the antigen; (*b*) bactericidal tests; (*c*) mouse protection tests; and (*d*) the ability of sera from immunized rabbits to induce long chain growth of streptococci. Antibodies to Group A polysaccharide and T protein were determined by capillary precipitin tests (18). All rabbits developed precipitins to M and T proteins as illustrated in Table III. Only sera from rabbit 1-1, immunized with the aid of adjuvant, developed demonstrable precipitin reactivity with purified Group A polysaccharide. In contrast to the relatively late appearance of type-specific antibody in natural immunity to human streptococcal infection (3, 4), antibody appeared in immunized rabbits early in the course of immunization and generally disappeared within 2 months.

Direct and indirect bactericidal tests were performed on all animals immunized with lysin M protein. The results of the direct bactericidal tests, using heparinized blood, are presented in Table IV. Of nine rabbits immunized, eight developed bactericidal properties 3 weeks after onset of immunization. Rabbit 1-7, immunized with lysin M protein in saline, demonstrated a poor immune

[14] Concentrated eluate from diethylaminoethyl (DEAE) cellulose column.

[15] Eluate purified by ammonium sulfate precipitation.

TABLE III

*Antibody Responses to Lysin M Protein Measured by Capillary Precipitin Technique**

Rabbit antiserum	Immunization		Acid M protein 0.1 per cent	T protein solution	Group A carbohydrate 50 γ/ml.
	Antigen used	Wks. after onset			
1-1	1.5 mg. crude‡ lysin M in adjuvant	2	±	ND	ND
		4	+	ND	ND
		6	+++	ND	±
		8	++++	ND	+
		10	+++	+++	++
		12	++	ND	+
1-2		2	±	ND	−
		3	+++	++	−
		4	++	ND	−
		6	+	ND	−
1-3	1.5 mg. crude‡ lysin M in saline	2	±	ND	−
		3	+++	++	−
		4	++	ND	−
		6	±	ND	−
1-4		2	±	ND	−
		3	++	+	−
		4	+	ND	−
		6	+	ND	−
1-5		2	±	ND	ND
		4	++	+	−
		7	+	±	−
		9	±	ND	ND
1-6	1.5 mg. lysin§ M in saline	2	−	ND	ND
		4	+	+	−
		5	++	±	−
		8	+	ND	ND
1-7		2	−	ND	−
		3	±	+	−
		4	−	ND	−
		6	−	ND	−
1-8		2	±	ND	−
		3	++	++	−
		4	+	ND	−
		6	±	ND	−
2-0		2	±	ND	−
		3	++	++	−
		4	+	ND	−
		6	+	ND	−

* Tubes read from ± to 4+ with increasing amounts of precipitate.

ND indicates determination not done.

‡ Material eluted in second peak from DEAE cellulose column.

§ Eluate purified by ammonium sulfate precipitation.

response in this, as well as other tests employed to determine the presence of type-specific antibody. The specificity of response to immunization, as measured by the more critical indirect bactericidal test, is demonstrated by the example of rabbit 1-1 (Table V). A positive result (with a bactericidal index that would be considered low, however, in the direct test) was obtained

TABLE IV

Bactericidal Properties of Rabbit Bloods against Type 1 Streptococci (T1/155/4) Following Immunization with Type 1 Lysin M Protein

Antigen used in immunization	Source of blood tested	Results of bactericidal tests, No. of colonies				Bactericidal index§
		Inoculum				
1.5 mg. crude* lysin M in adjuvant		165	40	6	5	
		Bactericidal tests				
	(*a*) Normal rabbit	∞	3000	580	240	
	(*b*) Immunized rabbit 1-1	52	17	0	0	200
		Inoculum				
1.5 mg. crude* lysin M in saline		250	76	17	1	
		Bactericidal tests				
	(*a*) Normal rabbit	∞	3500	600	24	
	(*b*) Immunized rabbit 1-2	16	1	1	0	611
	(*c*) Immunized rabbit 1-3	26	20	4	0	220
	(*d*) Immunized rabbit 1-4	200	54	0	0	45
		Inoculum				
		188	64	20	6	
		Bactericidal tests				
	(*a*) Normal rabbit	∞	1800	800	182	
	(*b*) Immunized rabbit 1-5	54	7	0	0	144
	(*c*) Immunized rabbit 1-6	71	26	1	0	90
1.5 mg. lysin‡ M in saline		*Inoculum*				
		195	53	10	1	
		Bactericidal tests				
	(*a*) Normal rabbit	∞	2500	500	90	
	(*b*) Immunized rabbit 1-7	1100	670	60	0	9
	(*c*) Immunized rabbit 1-8	11	2	8	0	787
	(*d*) Immunized rabbit 2-0	48	32	5	0	198

* Material eluted in second peak from DEAE cellulose column.

‡ Eluate purified by ammonium sulfate precipitation.

§ Bactericidal index is an expression for inhibition of growth of streptococci in the presence of homologous antibody compared with growth in control bloods in the absence of antibody (see Materials and Methods) $<25 = 0$; 25 to 50 = ±; 50 to 100 = 1+; 100 to 200 = 2+; 200 to 500 = 3+; >500 = 4+ inhibition.

∞ Indicates innumerable colonies.

only with the homologous type organism and not with streptococci of three heterologous types. Other data, not presented, demonstrated a similar and more marked effect in direct bactericidal tests using blood from rabbits 1-5 and 1-6.

TABLE V

Specificity of Bactericidal Properties of Antiserum from Rabbit 1-1

Strain tested	Conditions of test	Results of test No. of colonies				Bactericidal index
		Inoculum				
T1/155/4 (Type 1)	No. of streptococci inoculated	512	135	33	8	
	No. of streptococci at end of test:	*Bactericidal test*				
	(*a*) Normal rabbit serum	∞*	8000	2684	384	22.1
	(*b*) Rabbit 1-1 serum	1396	459	117	24	
		Inoculum				
S23/94/3 (Type 14)	No. of streptococci inoculated	326	106	34	8	
	No. of streptococci at end of test	*Bactericidal test*				
	(*a*) Normal rabbit serum	∞	8000	2032	884	2.6
	(*b*) Rabbit 1-1 serum	∞	∞	3137	605	
		Inoculum				
SF42 (Type 12)	No. of streptococci inoculated	322	75	28	9	
	No. of streptococci at end of test	*Bactericidal test*				
	(*a*) Normal rabbit serum	∞	3355	980	440	1.9
	(*b*) Rabbit 1-1 serum	∞	2997	960	333	
		Inoculum				
NCTC 8301 (Type 23)	No. of streptococci inoculated	293	72	18	4	
	No. of streptococci at end of test	*Bactericidal test*				
	(*a*) Normal rabbit serum	6000	1876	988	212	2.9
	(*b*) Rabbit 1-1 serum	6000	1508	794	36	

* For purposes of calculation, ∞ = 10,000.

Indirect bactericidal tests, using several different human donors, yielded similar results to those reported above. However, contrary to previous reports (8, 14), indirect tests with human donors were generally less sensitive than the corresponding direct tests.

Mouse protection tests were performed with the serum of rabbit 1-1, obtained 12 weeks after onset of immunization. White mice of the National Institutes of Health stock, weighing 15 to 20 gm., were injected with 0.5 ml of this serum intraperitoneally on the day before challenge: control mice received normal rabbit serum. Mice were challenged with 0.5 ml. each, intraperitoneally, of previously determined appropriate dilutions of 4 hour horse-meat infusion broth cultures of the homologous type and of three virulent heterologous types of Group A streptococci.

TABLE VI

Specific Protection of Mice by Serum from Rabbit No. 1-1 Immunized with Crude Type 1 Lysin M Protein

Serum 0.5 ml.	Group A streptococci tested	Culture dilution																													
		10^{-2}				10^{-3}				10^{-4}				10^{-5}				10^{-6}				10^{-7}				10^{-8}				10^{-9}			
		Mouse No.:				Mouse No.:				Mouse No.:				Mouse No.:				Mouse No.:				Mouse No:.				Mouse No.:				Mouse No.:			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Rabbit 1-1	T1/155/4 (Type 1)	1	1	1	1	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	—	—	—	—*	—	—	—	—	—	—	—	—
	T23(NCTC)8301 (Type 23)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	2	2	2	2	2	2	2	2	2	2	2	2	S	S	S
	S23/94/3 (Type 14)	—	—	—	—	—	—	—	—	—	—	—	—	1	1	1	1	2	2	2	S	2	2	2	2	2	2	S	S	—	—	—	—
	SF42 (Type 12)	—	—	—	—	—	—	—	—	—	—	—	—	1	1	1	1	1	1	1	1	1	1	1	1	1	2	S	S	—	—	—	—
Normal rabbit	T1/155/4 (Type 1)	1	1	1	1	1	2	2	S	1	1	1	S	1	1	S	S	1	S	S	S	—	—	—	—	—	—	—	—	—	—	—	—
	T23(NCTC)8301 (Type 23)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	2	2	1	2	2	2	2	2	2	2	2	S	S	S
	S23/94/3 (Type 14)	—	—	—	—	—	—	—	—	—	—	—	—	1	1	1	2	1	2	2	3	2	2	2	3	2	2	3	S	—	—	—	—
	SF42 (Type 12)	—	—	—	—	—	—	—	—	—	—	—	—	1	1	1	1	1	1	1	1	1	2	3	S	2	2	S	S	—	—	—	—

Numeral indicates day of death: "S" indicates survival for 10 days.

* —, Dilution not tested.

Table VI shows that the immune serum protected mice against between 100 and 1000 LD_{50}'s of the virulent Type 1 culture, and failed to protect against virulent streptococci of Types 12, 14, or 23.

To confirm the presence of M antibodies in the sera of immunized rabbits, growth of virulent Type 1 organisms in these sera was observed, with reference to chain length. For this purpose the method of Stollerman *et al.* was used (23).

TABLE VII

*Comparison of Chain Length of Streptococcus T1/SS70L1 Grown in Serum from Normal and Immunized Rabbits**

Rabbit serum	Antigen used in immunization	Chain length	
		Mean‡	Standard deviation
1-2	1.5 mg. crude lysin M in saline	14.9	±7.9
1-3		14.5	±7.9
1-4		7.8	±5.0
1-5	1.5 mg. lysin M in saline	19.7	±10.8
1-6		14.8	±8.0
1-7		7.8	±4.3
1-8		15.1	±8.5
2-0		14.3	±7.9
Normal serum§		3.4	±1.8

* Obtained 3 weeks after the onset of immunization.

‡ Mean of 30 chains counted.

§ Average of means derived from 29 normal rabbit sera. Each mean calculated on the basis of 30 chains counted.

An overnight culture of T1/SS70L1 was subcultured in Todd-Hewitt broth, and incubated at 37°C. to an optical density of 0.05 at 530 mμ in the Coleman Jr. spectrophotometer. The culture was then diluted 1:100 in broth, and 0.05 ml. was pipetted into a 7 × 100 mm. tube with 0.2 ml. of the serum to be tested. The tightly stoppered tube was incubated in a 37°C. water bath for 3.5 hours, and then gently inverted once before opening. The stopper was touched to the center of a clean glass slide, and a coverslip was carefully placed on the resulting drop. Thirty chains of streptococci were counted, and the mean chain length and standard deviation were calculated. To determine the mean chain length of this strain of streptococci (T1/SS70L1) grown in normal rabbit serum, 29 normal sera were tested, and 30 chains were counted in each serum. The mean chain length in normal sera varied from 2.3 to 4.9 cocci; the average of these mean chain lengths was 3.4 ± 1.8 cocci.

The mean chain lengths and standard deviations obtained from the sera of eight immunized rabbits are shown in Table VII. The chain lengths of streptococci grown in these sera were significantly longer than in normal sera ($p < 0.001$), except for Nos. 1-4 and 1-7 in which the level of significance is not as

great ($p < 0.01$). These results parallel those obtained in the precipitin and bactericidal tests.

In an additional test of specificity, the serum of rabbit 1-1 and of a normal rabbit were tested in the long chain reaction with streptococci of three heterologous types as well as with homologous type organisms. The results (Table VIII) confirm those of the bactericidal tests, indicating that the effect was type specific. (The heterologous types used, though not tested in their homologous antisera, were each M-rich, mouse-passed, and highly mouse-virulent strains: these properties have been shown (19) to be associated with ability to grow in long chains in homologous type antisera.)

TABLE VIII

Specificity of Serum from Rabbit 1-1 (Immunized with Crude Type 1 Lysin M Protein) in the Long Chain Reaction with Homologous and Heterologous Types of Group A Streptococci

Streptococcal strain	No. of cocci *per* chain when grown in:						Ratio of means§
	Rabbit 1-1 serum			Normal rabbit serum			
	Mean*	S.D.‡	Range	Mean	S.D.	Range	
T1/155/4 (Type 1)	27.8	±19.2	2–68	5.3	±3.0	2–14	5.2
S23/94/3 (Type 14)	4.1	±2.1	2–10	3.8	±1.9	2–8	1.1
SF42 (Type 12)	6.5	±4.1	2–20	10.1	±6.9	2–32	0.6
NCTC 8301 (Type 23)	4.8	±2.5	2–12	4.0	±2.4	2–10	1.2

* Average of 30 chains each.

‡ S.D. = standard deviation.

§ Equivalent to "long chain index" (23).

At the conclusion of these experiments all rabbits immunized with lysin M preparations were sacrificed and examined for evidence of toxicity. No gross or microscopic changes were found in the heart, lungs, liver, spleen, or kidneys.

DISCUSSION

In Group A streptococcal infections the relationship of M protein to virulence and immunity is well established. M antibodies are consistently demonstrated during the convalescent phase of human streptococcal infections, and the production of precipitin and bactericidal antibodies in the sera of rabbits following immunization with heat killed virulent organisms is assured (2–4). However, M protein extracted from streptococci in a boiling water bath at pH 2 is a poor antigen (5, 24). Barkulis and Jones (25) have estimated the trypsin-sensitive portion of Type 14 cell walls to be 40 per cent of the dry weight, but have recovered only 15 per cent of the dry weight in the form of M protein. It may be inferred that either the remaining 25 per cent of the trypsin-sensitive protein is not related to M protein or the harsh method of extraction results

in a haptene-like moiety which is only a fraction of a larger, more sensitive protein partly destroyed in the extraction.

The recent works of Maxted (6) and Krause (7) have made available an enzyme which attacks the carbohydrate framework of Group A streptococcal cell walls and releases, unaffected, the protein portions known to be sensitive to proteolytic enzymes. This enzyme, the phage-associated lysin, has been prepared from fresh phage lysates of Group C streptococci grown in a semisynthetic non-protein media. The media reported in the present paper supports growth of the propagating strain of Group C streptococci in a manner comparable to more complete protein-containing media and previously described dialysate media.

The site of action of the enzyme is unknown, except in so far as it results in a digest shown to contain the C polysaccharide and several protein constituents. Moreover, recently Krause (21) has determined that the polysaccharide released from the cell wall by phage-associated lysin is identical with that released by the enzymes obtained from streptomyces by McCarty (17). The physiochemical natures of the proteins released from the cell wall by phage-associated lysin are unknown. Electrophoretic determinations of lysin digests of Type 6 streptococcal cell walls in a starch supporting medium, reported by Krause, showed migration of the type-specific material toward the cathode at pH 8.7. The data presented here, collected on digests made from Type 1 organisms, indicates migration of lysin M protein towards the anode when the boundary electrophoresis is run at pH 8.6 in a veronal buffer. The use of different materials and methods permits neither comparison nor explanation of these diverse results.

The initial step in isolating the M protein from the digest is selective elution from a DEAE[16] cellulose ion-exchange column at pH 7 by increasing the ionic strength of the buffer. The ultraviolet absorption spectrum of the first peak to be removed from the column shows a flat curve in the 260 mμ region suggesting the presence of nucleic acids. No further purification of the material in this peak was attempted. It is interesting that the concentration of material reacting with Group A antiserum decreases with elution of the first peak, and then increases markedly with elution of the second peak containing the type-specific material. This suggests that some of the carbohydrate is bound to the protein, a suggestion which is strengthened by the continued ability of the lysin M protein to react with grouping antiserum after 2 precipitations with ammonium sulfate. The lysin M preparation has now been shown to contain T protein. It cannot be determined at this time whether the T protein is bound to the type-specific material or coexists as a separate entity. Lysin M protein did not react in the capillary tube precipitation test with heterologous group or type antisera. The inability to demonstrate the phage-associated lysin in either peak eluted from the ion-exchange column even though a 91 per cent recovery of protein was achieved, suggests that the process of chromatography irreversibly

[16] DEAE, diethylaminoethyl.

denatured the enzyme; it, too, may contribute to the electrophoretic inhomogeneity of the lysin M preparation.

At each of several salt concentrations (within the range of 0.01 to 0.6 M phosphate buffer) lysin M and acid M proteins reacted in different degree in the precipitin reaction with the same antiserum. The basis of this distinction in immunochemical properties is not known. Inhibition of the precipitin reaction between lysin M and absorbed antiserum at high salt concentrations may be due either to a solubility of the immune complex or inhibition of the formation of the antigen-antibody complex in these buffers.

The antigenicity of small amounts of lysin M protein when injected by the intradermal route was good. Precipitins were demonstrated to the M and T proteins, and in 1 rabbit, to the C polysaccharide. However, this rabbit was immunized with crude lysin M protein in complete Freund's adjuvant. The sera of all rabbits subsequently immunized without adjuvant failed to demonstrate reactivity with purified Group A polysaccharide.

Stollerman and Ekstedt (19) showed that the growth of streptococci in long chains in the presence of homologous antiserum is a function of the presence of type-specific antibody. The presence of M antibody in the sera of lysin-M-immunized rabbits was shown by the long chain reaction (Tables VII and VIII). Although not quantitatively comparable, the results of this test roughly paralleled relative antibody levels as determined for the same sera in the bactericidal test (Table IV). It has since been shown by Stollerman *et al.* (23) that the long chain reaction with human sera is specific and sensitive and correlates closely with the bactericidal test. Similarly, we have found that the long chain test is a convenient and reproducible means of determining the presence of type-specific antibody in rabbit sera.

The bactericidal efficiency of most rabbit bloods in the presence of antibody is relatively poor (8, 14). The strong direct bactericidal tests reported here, in the presence of minimal to moderate amounts of antibody, suggest that, as a group, the National Institutes of Health strain of white rabbits are more suitable as donors in the bactericidal test than those rabbits heretofore reported. That they are not unique in this donor property is demonstrated by the reports in two laboratories of occasional rabbits whose blood demonstrated strong bactericidal properties in the presence of antibody (8, 14).

The absence of local or systemic toxicity following intradermal inoculations of the lysin M preparation in rabbits encourages a trial immunization in human volunteers to produce type-specific immunity to streptococcal infection.

SUMMARY

The lysis of Group A type 1 streptococcal cell walls by phage-associated lysin has been described. In the preparation of lysin, a new semisynthetic nonprotein media to support growth of the propagating strain of Group C streptococci was employed. Following lysis of the cell walls, the resulting digest was

partially purified by ion-exchange chromatography and ammonium sulfate precipitation. In addition to M protein, the resulting preparation (called lysin M protein) contained the group-specific carbohydrate and the T protein—but did not contain antigens, detectable by precipitin tests, which cross-reacted with absorbed heterologous group or type antisera. Capillary precipitin reactions between the lysin M protein and type-specific antiserum did not occur in the presence of high ionic strength buffers; these buffers did not similarly affect precipitin reactions of acid M protein.

Type 1 lysin M protein is shown to be a good antigen. A total of 1.5 mg. injected intradermally in saline produced bactericidal antibody in eight of nine rabbits; when injected in adjuvant in one rabbit, protective serum antibodies developed. Streptococci grown in sera from seven of nine rabbits immunized with lysin M protein demonstrated significantly longer chains than when grown in normal rabbit serum. Antibody as demonstrated by each of these three tests was shown to be type-specific.

No local or systemic toxicity was noted following intradermal injection in rabbits of lysin M protein.

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